



**University of  
Zurich**<sup>UZH</sup>

**Zurich Open Repository and  
Archive**

University of Zurich  
University Library  
Strickhofstrasse 39  
CH-8057 Zurich  
[www.zora.uzh.ch](http://www.zora.uzh.ch)

---

Year: 2011

---

## **Biofilm reduction and staining potential of a 0.05% chlorhexidine rinse containing essential oils**

Hofer, D ; Meier, A ; Sener, B ; Guggenheim, B ; Attin, T ; Schmidlin, P R

Abstract: The test solution exhibited an antimicrobial activity. The composition, however, seems to hamper its effectiveness. Accordingly, it produced statistically significant, although by trend less, staining on restorative materials.

DOI: <https://doi.org/10.1111/j.1601-5037.2009.00437.x>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-50259>

Journal Article

Accepted Version

Originally published at:

Hofer, D; Meier, A; Sener, B; Guggenheim, B; Attin, T; Schmidlin, P R (2011). Biofilm reduction and staining potential of a 0.05% chlorhexidine rinse containing essential oils. *International Journal of Dental Hygiene*, 9(1):60-67.

DOI: <https://doi.org/10.1111/j.1601-5037.2009.00437.x>

**Biofilm reduction and staining potential of a 0.05% chlorhexidine  
rinse containing essential oils**

Deborah Hofer<sup>1</sup>, André Meier<sup>2</sup>, Beatrice Sener<sup>1</sup>, Bernhard Guggenheim<sup>2</sup>, Thomas  
Attin<sup>1</sup>, Patrick R. Schmidlin<sup>1</sup>

<sup>1</sup> Clinic for Preventive Dentistry, Periodontology and Cariology

<sup>2</sup> Institute for Oral Biology, Section for Oral Microbiology and General Immunology  
Center for Dental and Oral Medicine, University of Zurich, Plattenstrasse 11, 8032  
Zürich, Switzerland

Key words: Chlorhexidine, antimicrobial effectiveness, biofilm, discoloration, *in vitro*

Short title: Antimicrobial and anti-staining effect of CHX

\* Corresponding author:

PD Dr. Patrick Schmidlin, Clinic of Preventive Dentistry, Periodontology and  
Cariology, Center for Dental and Oral Medicine and Maxillofacial Surgery  
University of Zürich  
Plattenstrasse 11  
8032 Zürich  
Switzerland

Tel +41 44 634 08 46

Fax +41 44 634 43 08

Email [patrick.schmidlin@zzmk.uzh.ch](mailto:patrick.schmidlin@zzmk.uzh.ch)

## Abstract

*Objectives:* To assess the biofilm reduction and discoloration potential of a new 0.05% chlorhexidine (CHX) digluconate solution, containing additional essential oil and alcohol components (Parodontosan®), as compared to two control CHX solutions (0.05% CHX and 0.2% CHX) and water.

*Methods:* The potential to reduce total viable counts of growing mixed microbial populations was examined using the Zurich biofilm model. Biofilms were created on sterile pellicle-coated hydroxyapatite discs and were exposed to test substances at different time points. After 64.5 h, mean CFU (colony forming units) and standard deviations were determined. Color change measurements using light reflection analysis were carried out on saliva pre-conditioned bovine dentin and enamel samples, as well as composite and a glass ceramic restorative materials, after successive immersions in a standardized tea brew and the CHX solutions.

*Results:* Parodontosan was able to reduce biofilm formation by 3 log steps as compared to the water control. This was significantly less effective than the control CHX solutions, which were able to reduce viable counts by 6 log steps. Both Parodontosan and the control rinses exhibited staining on all surfaces. Staining was most pronounced on dentin, followed by enamel and to a significantly lesser degree on the restorative materials. The staining caused by Parodontosan was generally lower than by the control solutions on the restorative materials.

*Conclusions:* Parodontosan exhibited an antimicrobial activity. The composition, however, seems to hamper its effectiveness. Accordingly, it produced statistically significant, though by trend less, staining on restorative materials.

(243 words)

## Introduction

Various species of bacteria found in mature dental biofilms are recognized contributing factors to both periodontal diseases and caries development (1, 2). It has been shown that meticulous daily plaque control will prevent disease initiation, stop progression of the disease process, and combined with professional debridement, allow surrounding tissues to return to a healthy state (3-5). Unfortunately, inadequate daily removal of bacterial plaque and biofilm is widespread (6-8). Even well trained patients may miss hard-to-reach areas around posterior teeth or marginal gingiva. Additionally, people with malpositioned teeth, bridgework or orthodontic appliances and especially elderly people with physical or mental limitations may find brushing and interdental cleaning extremely difficult (9). Antimicrobial rinses are therefore often recommended as an adjunctive homecare procedure. Of all antimicrobials studied and currently used, chlorhexidine (CHX) has long been recognized as being the most effective for inhibiting plaque, preventing gingivitis and displaying a well-documented anti-caries effect (10-13). In repeated studies, depending upon the concentration used, chlorhexidine has been shown to prevent plaque accumulation, with two marked negative side effects: surface staining and altered taste perception (14). Both side effects are reversible upon discontinuation of use, but remain a major stumbling block in regard to patient compliance.

Researchers, and industry, have placed a lot of effort in developing formulations that reduce the negative side effects while maintaining the powerful antimicrobial effect of chlorhexidine. However, due to the strong positive charge, CHX loses its antimicrobial effect rapidly when combined with organic or un-organic molecules (15, 16). Only lower concentrations appear to cause less stain, or less rapid staining, but at the cost of efficacy (17-19).

This study was designed and executed in two-parts, to determine first the antimicrobial efficacy of a new 0.05% chlorhexidine digluconate solution containing essential oil and alcohol components (Parodontosan®, Tetan AG, Ramllinsburg, Switzerland) and then assess its staining potential compared to control solutions. The null hypothesis tested was that the test product I) is as effective in reducing biofilm formation as the control solutions and II) that that it will cause identical staining as control solutions on enamel, dentin and selected restorative materials.

## Study population and methodology

### Antimicrobial solutions

The solutions tested in this experiment are listed in Table 1. Parodontosan is readily available over the counter in Switzerland. The positive control chlorhexidine solutions were prepared in-house to ensure purity. Deionized water was used as a negative control.

### Experiment 1: Biofilm formation

#### Biofilms

Biofilms contained *Actinomyces naeslundii* OMZ 745, *Veillonella dispar* OMZ 493, *Fusobacterium nucleatum* OMZ 596, *Streptococcus sobrinus* OMZ 176, and *Streptococcus oralis* OMZ 607 and *C. albicans* OMZ 110. Pellicle-coated hydroxyapatite discs (Ø 10.6 mm) in 24-well polystyrene cell culture plates were covered with 1.6 ml of processed whole unstimulated saliva + modified fluid universal medium (mFUM), supplemented with 67 mmol/l Sørensen's buffer (38% v/v), final pH 7.2) containing carbohydrate (20). The carbohydrate concentration in stock solutions of mFUM was 0.3% (w/v) and consisted of either glucose (biofilm cultivation from 0 to 16.5 h) or a 1:1 (w/w) mixture of glucose and sucrose (biofilm cultivation from 16.5 to 64.5 h). Wells were inoculated with mixed cell suspensions (200 µL) prepared from equal volumes of each species adjusted to an OD 550 and incubated anaerobically at 37° C. Medium was changed after dipping (see below) at 16.5 and 40.5 h by aspirating spent medium and adding back fresh medium (21).

#### Evaluation of Antimicrobial Activities of Test Solutions

Biofilm-covered discs were immersed for 1 min in 1 ml of test solution, and then rinsed gently by dipping in physiological saline (3 x 2 ml). Biofilms were exposed to test substances at 16.5, 20.5, 24.5, 40.5, 44.5 and 48.5 h. After the last treatment biofilms were incubated undisturbed and harvested at 64.5 h by vigorous vortexing in physiological saline (1 ml).

Aliquots of harvested biofilm were sonified, diluted, and spiralplated onto Columbia agar base (Oxoid, Ltd., Basingstoke, Hamps., UK) containing 5% (v/v) haemolysed human blood (CBA) and incubated anaerobically at 37° C. Colony-forming units (CFUs) were counted 72 h after plating with the aid of a stereomicroscope.

## Statistics

Statistical analyses of the effects of different treatments on total biofilm populations were performed using log<sub>10</sub>-transformed *total* CBA CFUs. Skew distributions of the values measured for most products and different variances of the solutions examined required non-parametrical statistical tests. Overall statistical analyses within defined groups of products were performed using the Kruskal-Wallis procedure as implemented in the program StatView II (Abacus Concepts, Inc., Berkeley CA, USA). Due to the multiple test situation, Bonferroni's correction was applied. Results are presented as boxplots.

## Experiment 2: Staining potential

### Stain formation

A standardized *in vitro* method for reproducing stain in the presence of CHX was followed (22). A standard tea solution (Marks and Spencer extra strong, London, UK) was prepared by boiling 8g in 800 ml of distilled water for 2 minutes. The solution was allowed to cool in a refrigerator at 4°C for 30 minutes and the infusion filtered through gauze to remove the tealeaves. Finally the tea solution was kept at 37°C during the experiment.

As test specimens, this investigation used different tooth and restorative materials (Table 2), in place of clear acrylic blocks. These specimens were prepared as follows: the crowns from sixty-four caries-free bovine mandibular incisors of two and a half year old animals were mechanically separated, the labial aspects sectioned (enamel n=32), and the middle dentine (n=32) prepared using a PD-Max grinder (Streuers GmbH, Birmensdorf, Switzerland) at 300 revolutions per minute, under water cooling with SiC paper 500 grit (Merck, Dietikon, Switzerland) followed by p1000 grit (DIN 69176; grit size 18 µm) wet and polished by hand to a standardized reproducible flat surface [ISO/TR 1994]. The restorative materials were prepared by placing each material (n=32/material) in 13 mm round, 3 mm thick Teflon forms. In a first curing phase, the forms were only half filled and cured with a UV light source (blue phase, Ivoclar, Schaan, Liechtenstein) on 4 points within the circle radius for a total of 40 seconds. A second curing phase was performed after the forms were fully filled, on 6 points, for a total of 60 seconds. The specimens were then placed in a broad beam light-curing chamber (Spectramat, Ivoclar, Schaan, Liechtenstein) and cured for a third time, for 5 minutes.

All specimens were embedded in optically clear epoxy resin (Stycast®, Emerson & Cuming, Waterloo, Belgium), which was mixed in the proportion Stycast 1266 Part A 15 g,

Part B 4.2 g, 90 s by hand then 19 min under a vacuum pump to generate a bubble-free mass. The outer dimensions of the samples measured 20 mm in diameter, to fit the optical lens of a Konica/Minolta spectrophotometer (CM-508d, Konica Minolta Photo Imaging (Schweiz) AG, Switzerland).

The 5 specimen materials were divided into 4 groups of 8 specimens each. They were covered and bathed in a pooled stimulated human saliva (gathered from volunteers at 7.45 and 11.45 on the day of testing, with no food having been ingested for at least 2 hours previously and held between cycles at 37°C) for 2 min at 37°C, then rinsed 4 times consecutively with 2 ml deionised water. Each eight specimens were then covered and bathed by groups in one of the three CHX solutions applied in this experiment (Table 1) or with a deionised water negative control at 37°C for another 2 min, before being rinsed again 4 times consecutively with 2 ml deionised water. The specimens were then covered and bathed in the standard tea solution and reincubated for 1 h at 37°C. A final rinse of 4 consecutive 2ml washes was performed and the specimens were dried with compressed air and measured for luminosity using the CIELAB (L\*a\*b\*) color system on a daylight, D65/10°, scale. The saliva/CHX/tea bath cycle was repeated 6 times over 11 hours.

### **Stain determination**

A baseline L\*a\*b\* reading had been taken prior to the start of the saliva/chlorhexidine/tea baths and the changes in L\* (luminosity), a\* (red-green axis) and b\* (yellow-blue axis) were recorded by a Konica/Minolta spectrophotometer (CM-508d, wavelength range 400-700nm) and fed directly into a computer (MacIntosh G4, Apple, Cupertino, CA, USA).

The overall color difference was calculated as:

$$\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

where  $\Delta L^* = L^*_{\text{interval}} - L^*_{\text{baseline}}$ ;  $\Delta a^* = a^*_{\text{interval}} - a^*_{\text{baseline}}$ ;

$\Delta b^* = b^*_{\text{interval}} - b^*_{\text{baseline}}$

### **Statistics**

Two-way analysis of variance (ANOVA) was used to compare differences in color upon the various substrate materials and under the influence of the 3 different chlorhexidine solutions and deionized water control. Means were compared with Scheffe's multiple comparison test at the 0.05 level of significance (StatView, Abacus Concepts, Inc., Berkley CA, USA).

## Results

### *Experiment 1*

The results of the biofilm experiment are graphically depicted in Figure 1.

The water control showed the highest number of viable microorganisms ( $1.5 \times 10^8 \pm 5.3 \times 10^7$ ). Parodontosan was able to reduce the biofilm growth by 3 log steps ( $6.4 \times 10^5 \pm 1.1 \times 10^6$ ;  $p < 0.05$ ). The CHX control solutions showed almost complete inhibition of bacterial growth, i.e. a reduction of 6 and 7 log steps respectively.

### *Experiment 2*

The results are summarized in Table 3.

All substrate surfaces tended to become darker and more discolored over time. This darkening ( $\Delta L^*$ ) and discoloration ( $\Delta E^*$ ) was significantly more pronounced on the enamel and dentin samples, as compared to the 3 restorative materials. While contact with the test solutions did not produced significantly more staining than contact with water before the tea bath on either the enamel or dentin specimens, the change in luminosity ( $\Delta L^*$ ) caused by the 0.05% CHX solution was significantly higher than either the 0.2% CHX solution or Parodontosan. For the dentin samples, the color change along the red/green axis ( $\Delta a^*$ ) was significantly greater for both pure CHX solutions than the change displayed by Parodontosan. However, there were no significant changes in luminosity or overall color ( $\Delta E^*$ ) for these dentin substrate samples, when compared to those caused by the water control.

On the micro-filler and ceramic substrates, the level of darkening ( $\Delta L^*$ ) was significantly greater after contact with the pure CHX solutions, as compared to water or Parodontosan. However, all 3 CHX solutions produced a level of staining significantly higher than the water control on the nano-filler, with Parodontosan showing a tendency, though not statistically significant, for lesser staining also on this substrate.

On the nano-filler and ceramic specimens, significant shifts in overall color change ( $\Delta E^*$ ) occurred after exposure to both the additive and pure CHS solutions. Again, Parodontosan displayed a tendency, though not statistically significant, for less staining. However, on the micro-filler substrate, staining was significant for those specimens exposed to the pure CHX solutions, while Parodontosan only displayed a statistically insignificant difference to that recorded by the specimens exposed to the water control.



## Discussion

This study assessed the capability of a new 0.05% chlorhexidine digluconate solution containing essential oil (EO) and alcohol components to inhibit biofilm formation, as well as to determine its staining potential as compared to standard solutions.

It was found that the test solution had a significant antibacterial effect on the experimental biofilm, however a better efficiency with pure CHX solutions with or without equimolar alcohol content was observed. The latter solutions almost completely prevented biofilm formation and reduced the biofilm growth by 6 to 7 log steps. Therefore, the first null hypothesis was rejected.

As compared to data of a previous study applying the same methodology, the efficiency of the test product can be compared to Listerine® (Johnson & Johnson Healthcare Products, Skillman, NJ, USA), which contains menthol, thymol, methyl salicylate, and eucalyptol (23). Parodontosan and Listerine both reduce biofilm formation by 3 log steps. In Listerine, ethanol is present in concentrations of 21.6% in the flavored products and 26.9% in the original antiseptic formulation. Parodontosan contains 15 volume percent.

For the biofilm formation, hydroxyapatite discs were chosen to simulate the enamel structure. A previous study assessed biofilm growth on other substrates, i.e. human enamel and different composite resin materials (24). That study showed that surface roughness influenced initial biofilm adherence during the initial adherence phase (20 minutes), but differences vanished following growth and maturation (16.5 h).

The staining experiment showed that test and control rinses exhibited staining on all surfaces. Staining was most pronounced on dentin, followed by enamel and to a significantly lesser degree on restorative materials. The staining caused by the 0.05% CHX rinse containing EO was generally lower than the control CHX solutions on the restorative materials. In terms of the materials selected in this experiment, substrates relevant for the oral environment under clinical conditions, e.g. enamel, dentin, composite resin and ceramic material, were chosen.

Bovine enamel and dentin samples are commonly used as proxies for their human counterparts, as they are readily attainable in sufficient quantities for a study of this nature. The three restorative materials chosen for inclusion in this study were representative of their class: a micro filler hybrid, a nano filler hybrid, and a glass ceramic. All surfaces were polished with the same grit (P1000) to obtain comparable surfaces. As Stober et al. (2001) pointed out, this does not necessarily represent a clinical level of polishing, which might have reduced the level of staining produced (25). However, it does provide a standard, and

observable, level to which the finding of this study can be compared to the findings of other studies.

After polymerization shrinkage (in the case of micro filler and nano filler hybrids) and secondary caries, plaque accumulation and color stability are most often mentioned as major problems in tooth-colored restorations. Since chlorhexidine is often prescribed for patients with both an elevated caries risk and persistent periodontal problems, the possibility or probability of staining takes on a certain level of importance.

Stober et al. (2001) also offer a review of 6 studies evaluating the level of overall color change ( $\Delta E^*$ ) that can be detected by the human eye (25). Their conclusion, which is consistent with Fay et al. (1999), Abu-Bakr et al. (2000), and Lee & Powers (2004), is that a value of greater than 3.3 is visible to the human eye and must be considered unacceptable (26-28).

Based on these parameters, the results of the present study show that contact with saliva and tea alone was enough to cause clinically unacceptable color changes on enamel, dentin, micro-filler, nano-filler and ceramic restorative materials. This finding was unexpected and is in contrast to findings of other studies that found significantly less staining when specimens are subjected to saliva, water and tea than when CHX is added to the submersion protocol (29, 30). However Carpenter et al. (2005) also found greater staining on their specimens (hydroxyapatite discs pre-treated with parotid saliva to form an acquired pellicle) when exposed to tea alone than when exposed to CHX and tea together (31). Thus, there is no consensus concerning this issue. Methodological aspects in terms of substrate to be stained, pellicle formation and teas used and immersion protocols may cause differences in the outcomes.

The second null hypothesis, that Parodontosan will stain, as measured by overall color change, as heavily as an equivalently dosed non-additive CHX solution was confirmed on a statistical level on all test substrates except the micro-filler. There was, however, a displayed tendency to lesser overall color change on the other 2 restorative materials as well as the enamel and dentin specimens. Both the micro-filler and ceramic restorative materials also displayed statistically less darkening ( $\Delta L^*$ ).

This tendency toward lesser staining/darkening by essential oil compounds has also been observed in at least two comparative trials between CHX and Listerine (32, 33). The additives in Parodontosan, menthol, myrrh and sage, are similar to those found in Listerine, where they have been proven to be clinically effective anti-gingivitis and, though to a lesser degree, anti-plaque agents that do not promote extrinsic tooth stain (33, 34). So while their inclusion in

Parodontosan inactivated the CHX to a significant degree, as shown in the biofilm experiment, the mouth rinse still displays a statistical significance when compare to the water control in reducing biofilm growth.

In general, it must be remembered that the staining protocol, as explained in the Methods section, was designed to provide maximum staining potential in an *in vivo* setting. As such, it has a restricted applicability to a clinical situation where different types of tea (in different concentrations, temperatures, possibly the presence of milk) was well as other dietary chromogens and the influence of tooth brushing and tooth pastes all play a role in the accumulation of extrinsic stains (19, 27). However, such protocols do allow comparisons between test solutions and test substrates, and point out staining tendencies that might warrant further investigation.

An unexpected observation was that the 0.05% non-additive CHX solution produced both overall color change and darkening to the same order of magnitude as the 0.2% solution. CHX has been shown over the years and in many studies to be dose dependent (35-37). However, a careful analysis of previous dose and staining trials revealed one study that provided a similar result, whereby a 0.1% CHX solution stained significantly more than a 0.2% solution (19). The authors of this study did not address this result in their discussion of their findings. Here, too, the factors behind this result are not clear. Possible explanations include mechanisms of competitive binding to the pellicle, saturation of receptors or changes in valency of the dicationic molecule. Further research is needed to clarify this issue.

## **Conclusions**

The 0.05% chlorhexidine digluconate solution containing EO and alcohol components (Parodontosan) under investigation showed a significant reduction of biofilm formation. However, this action was less pronounced than the pure CHX controls with or without alcohol. The test solution displayed significantly less staining on a micro-filler composite restorative material than either a 0.05% or 0.2% non-additive CHX solution. It caused, however, an overall color change on enamel, dentin, a nano-filler composite and ceramic restorative materials. While not statistically significant, it was slightly less pronounced than the staining caused by the non-additive CHX solutions tested. In general, the restorative materials displayed significantly less color change and loss of luminosity after having been bathed in saliva, CHX and tea than did the enamel and dentin substrates tested under the same conditions.

## **Acknowledgement**

This research project was supported by Tetan AG, Ramllinsburg, Switzerland.

Table 1 Test and control solutions		
Type	Brand name	Manufacturer
CHX 0.05% with sage, menthol, myrrhe and 15 Vol.% ethanol	Parodontosan	Tentan AG, Ramlinsburg, Switzerland
CHX 0.05% non-additive	(produced in-house)	Kantonsapotheke Zurich, Switzerland
CHX 0.2% non-additive	(produced in-house)	Kantonsapotheke Zurich, Switzerland
Deionized H <sub>2</sub> O	(produced in-house)	Kantonsapotheke Zurich, Switzerland
Tea	Marks and Spencer extra strong	Marks and Spencer London, UK

353  
354  
355  
356  
357  
358  
359  
360  
361  
362  
363  
364  
365  
366  
367  
368  
369  
370  
371  
372  
373  
374  
375  
376  
377  
378  
379  
380  
381  
382  
383  
384  
385  
386

387

Table 2 Study specimens		
Type	Brand name	Manufacturer
Enamel	-----	-----
Dentin	-----	-----
Micro filler	Tetric A2	Ivoclar, Schaan, Liechtenstein
Nano filler	Filtek Supreme XT A2B	3M ESPE, Seefeld, Germany
Glass ceramic	Empress CAD A2	Ivoclar, Schaan, Liechtenstein

388  
389  
390  
391  
392  
393  
394  
395  
396  
397  
398  
399  
400  
401  
402  
403  
404  
405  
406  
407  
408  
409  
410  
411  
412  
413  
414  
415  
416  
417  
418  
419  
420  
421  
422  
423  
424  
425  
426

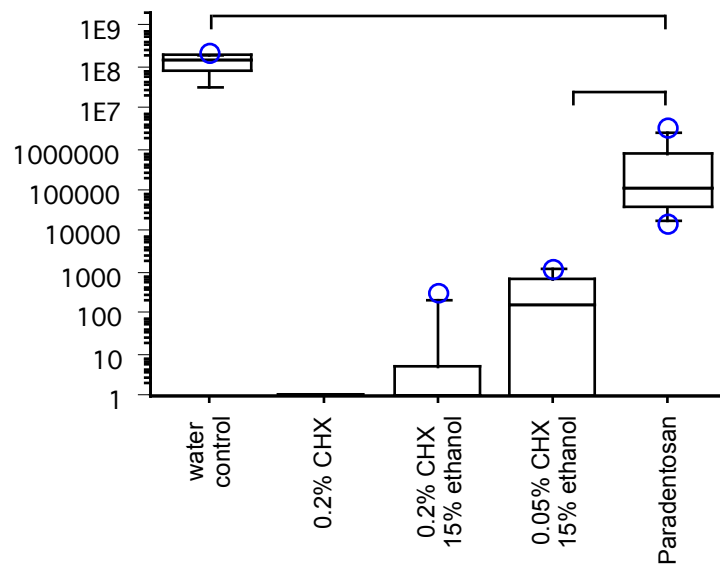


Fig. 1. Colony-forming units (CFUs) after exposure to test substances

\* indicates statistical significance at  $p < 0.05$

462  
463

Table 3 Mean Changes in luminosity, color, chroma and hue ( $\pm$ one standard deviation)					
Identical upper case letters (to be read horizontally) indicate a significant difference					
		Water	CHX 0.05	CHX 0.2	Paradentosan
Enamel	$\Delta L$	$-8.5 \pm 3.5$	$-10.1 \pm 0.7^{AB}$	$-8.9 \pm 2.7^A$	$-8.6 \pm 2.2^B$
	$\Delta a$	$6.1 \pm 1.9$	$6.8 \pm 0.8$	$7.5 \pm 1.1$	$6.7 \pm 1.0$
	$\Delta b$	$12.6 \pm 3.6$	$14.4 \pm 1.2$	$15.4 \pm 1.8$	$13.2 \pm 1.1$
	$\Delta E$	$16.5 \pm 4.8$	$18.8 \pm 1.1$	$19.5 \pm 2.3$	$17.3 \pm 2.2$
	$\Delta C$	$14.0 \pm 3.9^A$	$15.9 \pm 1.4$	$18.0 \pm 2.1^A$	$14.8 \pm 1.4$
	$\Delta H$	$23.4 \pm 6.8$	$26.6 \pm 1.6$	$28.1 \pm 3.4$	$24.5 \pm 3.1$
Dentin	$\Delta L$	$-11.3 \pm 4.0$	$-15.0 \pm 2.9$	$-14.0 \pm 2.8$	$-10.9 \pm 3.1$
	$\Delta a$	$7.1 \pm 1.5^{AB}$	$9.8 \pm 1.5^A$	$9.6 \pm 1.4^B$	$8.5 \pm 1.9$
	$\Delta b$	$15.0 \pm 3.0$	$16.9 \pm 3.3$	$17.0 \pm 2.7$	$13.9 \pm 4.5$
	$\Delta E$	$20.5 \pm 3.1$	$24.7 \pm 4.0$	$24.2 \pm 3.1$	$19.8 \pm 5.1$
	$\Delta C$	$16.7 \pm 2.8$	$19.6 \pm 3.0$	$19.6 \pm 2.5$	$16.4 \pm 4.4$
	$\Delta H$	$29.0 \pm 4.4^A$	$35.0 \pm 5.8^B$	$34.2 \pm 4.3^C$	$7.5 \pm 2.9^{ABC}$
Composite (micro filler)	$\Delta L$	$-3.0 \pm 1.7^{AB}$	$-5.8 \pm 1.0^A$	$-5.6 \pm 0.5^B$	$-4.0 \pm 2.2$
	$\Delta a$	$1.7 \pm 0.9^{AB}$	$3.7 \pm 0.4^A$	$3.6 \pm 0.4^B$	$2.5 \pm 1.6$
	$\Delta b$	$3.9 \pm 1.1^{AB}$	$7.9 \pm 0.8^A$	$8.3 \pm 0.6^B$	$5.8 \pm 1.6^B$
	$\Delta E$	$5.2 \pm 2.0^{AB}$	$10.5 \pm 1.1^{AC}$	$10.6 \pm 0.7^{BD}$	$7.5 \pm 2.0^{CD}$
	$\Delta C$	$4.2 \pm 1.4^{AB}$	$8.7 \pm 0.8^A$	$9.0 \pm 0.7^B$	$6.3 \pm 2.1^A$
	$\Delta H$	$7.4 \pm 2.9^{AB}$	$14.8 \pm 1.6^{AC}$	$15.0 \pm 1.10^{BD}$	$10.6 \pm 4.2^{CD}$
Composite (nano filler)	$\Delta L$	$-3.0 \pm 0.9^{ABC}$	$-5.6 \pm 0.7^A$	$-5.3 \pm 0.8^B$	$-4.3 \pm 1.6^C$
	$\Delta a$	$1.8 \pm 0.5^{ABC}$	$3.6 \pm 0.6^A$	$3.6 \pm 0.6^B$	$2.9 \pm 1.1^C$
	$\Delta b$	$3.4 \pm 0.8^{AB}$	$7.4 \pm 0.9^A$	$7.4 \pm 0.8^B$	$5.7 \pm 1.1^B$
	$\Delta E$	$4.9 \pm 1.2^{ABC}$	$9.9 \pm 1.2^A$	$9.8 \pm 1.1^B$	$7.9 \pm 2.1^C$
	$\Delta C$	$3.9 \pm 0.9^{AB}$	$8.2 \pm 0.9^A$	$8.3 \pm 0.9^B$	$6.4 \pm 1.5^A$
	$\Delta H$	$6.9 \pm 1.7^{ABC}$	$14.0 \pm 1.6^A$	$13.9 \pm 1.5^B$	$11.2 \pm 3.5^C$
Ceramic	$\Delta L$	$-2.8 \pm 2.0^{AB}$	$-5.5 \pm 0.8^A$	$-4.9 \pm 0.7^B$	$-4.6 \pm 1.6$
	$\Delta a$	$1.9 \pm 1.0^{ABC}$	$3.7 \pm 0.8^A$	$3.5 \pm 0.5^B$	$3.5 \pm 0.6^C$
	$\Delta b$	$3.1 \pm 1.2^{ABC}$	$6.7 \pm 1.2^A$	$6.3 \pm 1.4^B$	$6.0 \pm 0.5^C$
	$\Delta E$	$4.9 \pm 1.2^{ABC}$	$9.4 \pm 1.5^A$	$8.8 \pm 1.5^B$	$8.4 \pm 0.8^C$
	$\Delta C$	$3.6 \pm 1.5^{ABC}$	$7.6 \pm 1.4^A$	$7.2 \pm 1.5^B$	$6.9 \pm 0.6^C$
	$\Delta H$	$6.6 \pm 3.4^{ABC}$	$13.4 \pm 2.2^A$	$12.4 \pm 2.1^B$	$11.9 \pm 1.2^C$

464  
465  
466  
467  
468  
469  
470  
471  
472



## References

- 1 Listgarten MA. Structure of the microbial flora associated with periodontal health and disease in man. A light and electron microscopic study. *J Periodontol* 1976; **47**: 1-18.
- 2 Paster BJ, Boches SK, Galvin JL et al. Bacterial diversity in human subgingival plaque. *J Bacteriol* 2001; **183**: 3770-3783.
- 3 Lindhe J, Okamoto H, Yoneyama T, Haffajee A, Socransky SS. Longitudinal changes in periodontal disease in untreated subjects. *J Clin Periodontol* 1989; **16**: 662-670.
- 4 Loe H, Theilade E, Jensen SB. Experimental Gingivitis in Man. *J Periodontol* 1965; **36**: 177-187.
- 5 Loos B, Claffey N, Crigger M. Effects of oral hygiene measures on clinical and microbiological parameters of periodontal disease. *J Clin Periodontol* 1988; **15**: 211-216.
- 6 Addy M, Dummer PM, Griffiths G, Hicks R, Kingdon A, Shaw WC. Prevalence of plaque, gingivitis and caries in 11-12-year-old children in South Wales. *Community Dent Oral Epidemiol* 1986; **14**: 115-118.
- 7 De la Rosa M, Zacarias Guerra J, Johnston DA, Radike AW. Plaque growth and removal with daily toothbrushing. *J Periodontol* 1979; **50**: 661-664.
- 8 Lavstedt S, Modeer T, Welande E. Plaque and gingivitis in a group of Swedish schoolchildren with special reference to toothbrushing habits. *Acta Odontol Scand* 1982; **40**: 307-311.
- 9 Baker K. Mouthrinses in the prevention and treatment of periodontal disease. *Curr Opin Periodontol* 1993; 89-96.
- 10 Flotra L, Gjermo P, Rolla G, Waerhaug J. A 4-month study on the effect of chlorhexidine mouth washes on 50 soldiers. *Scand J Dent Res* 1972; **80**: 10-17.
- 11 Loe H, Schiott CR. The effect of mouthrinses and topical application of chlorhexidine on the development of dental plaque and gingivitis in man. *J Periodontal Res* 1970; **5**: 79-83.
- 12 Kidd EA. Role of chlorhexidine in the management of dental caries. *Int Dent J* 1991; **41**: 279-286.
- 13 van Rijkom HM, Truin GJ, van 't Hof MA. A meta-analysis of clinical studies on the caries-inhibiting effect of chlorhexidine treatment. *J Dent Res* 1996; **75**: 790-795.
- 14 Schiott CR, Loe H, Jensen SB, Kilian M, Davies RM, Glavind K. The effect of chlorhexidine mouthrinses on the human oral flora. *J Periodontal Res* 1970; **5**: 84-89.

- 507 15 Addy M. Chlorhexidine compared with other locally delivered antimicrobials. A short  
508 review. *J Clin Periodontol* 1986; **13**: 957-964.
- 509 16 Jenkins S, Addy M, Wade W. The mechanism of action of chlorhexidine. A study of  
510 plaque growth on enamel inserts in vivo. *J Clin Periodontol* 1988; **15**: 415-424.
- 511 17 Addy M, Wade WG, Jenkins S, Goodfield S. Comparison of two commercially  
512 available chlorhexidine mouthrinses: I. Staining and antimicrobial effects in vitro. *Clin*  
513 *Prev Dent* 1989; **11**: 10-14.
- 514 18 Addy M, Wade W, Goodfield S. Staining and antimicrobial properties in vitro of some  
515 chlorhexidine formulations. *Clin Prev Dent* 1991; **13**: 13-17.
- 516 19 Addy M, Mahdavi SA, Loyn T. Dietary staining in vitro by mouthrinses as a  
517 comparative measure of antiseptic activity and predictor of staining in vivo. *J Dent*  
518 1995; **23**: 95-99.
- 519 20 Gmur R, Guggenheim B. Antigenic heterogeneity of *Bacteroides intermedius* as  
520 recognized by monoclonal antibodies. *Infect Immun* 1983; **42**: 459-470.
- 521 21 Guggenheim B, Giertsen E, Schupbach P, Shapiro S. Validation of an in vitro biofilm  
522 model of supragingival plaque. *J Dent Res* 2001; **80**: 363-370.
- 523 22 Prayitno S, Addy M. An in vitro study of factors affecting the development of staining  
524 associated with the use of chlorhexidine. *J Periodontal Res* 1979; **14**: 397-402.
- 525 23 Shapiro S, Giertsen E, Guggenheim B. An in vitro oral biofilm model for comparing the  
526 efficacy of antimicrobial mouthrinses. *Caries Res* 2002; **36**: 93-100.
- 527 24 Dezelic T, Guggenheim B, Schmidlin PR. Multi-species biofilm formation on dental  
528 materials and an adhesive patch. *Oral Health Prev Dent* 2009; **7**: 47-53.
- 529 25 Stober T, Gilde H, Lenz P. Color stability of highly filled composite resin materials for  
530 facings. *Dent Mater* 2001; **17**: 87-94.
- 531 26 Fay RM, Servos T, Powers JM. Color of restorative materials after staining and  
532 bleaching. *Oper Dent* 1999; **24**: 292-296.
- 533 27 Abu-Bakr N, Han L, Okamoto A, Iwaku M. Color stability of compomer after  
534 immersion in various media. *J Esthet Dent* 2000; **12**: 258-263.
- 535 28 Lee YK, Powers JM. Discoloration of dental resin composites after immersion in a  
536 series of organic and chemical solutions. *J Biomed Mater Res B Appl Biomater* 2005;  
537 **73**: 361-367.
- 538 29 Addy M, Moran J, Newcombe R, Warren P. The comparative tea staining potential of  
539 phenolic, chlorhexidine and anti-adhesive mouthrinses. *J Clin Periodontol* 1995; **22**:  
540 923-928.

541 30 Addy M, Sharif N, Moran J. A non-staining chlorhexidine mouthwash? Probably not: a  
542 study in vitro. *Int J Dent Hyg* 2005; **3**: 59-63.

543 31 Carpenter GH, Pramanik R, Proctor GB. An in vitro model of chlorhexidine-induced  
544 tooth staining. *J Periodontal Res* 2005; **40**: 225-230.

545 32 Moran J, Pal D, Newcombe R, Addy M. Comparison of a phenolic and a 0.2%  
546 chlorhexidine mouthwash on the development of plaque and gingivitis. *Clin Prev Dent*  
547 1991; **13**: 31-35.

548 33 Charles CH, Mostler KM, Bartels LL, Mankodi SM. Comparative antiplaque and  
549 antigingivitis effectiveness of a chlorhexidine and an essential oil mouthrinse: 6-month  
550 clinical trial. *J Clin Periodontol* 2004; **31**: 878-884.

551 34 Overholser CD, Meiller TF, DePaola LG, Minah GE, Niehaus C. Comparative effects of  
552 2 chemotherapeutic mouthrinses on the development of supragingival dental plaque and  
553 gingivitis. *J Clin Periodontol* 1990; **17**: 575-579.

554 35 Cumming BR, Loe H. Optimal dosage and method of delivering chlorhexidine solutions  
555 for the inhibition of dental plaque. *J Periodontal Res* 1973; **8**: 57-62.

556 36 Jenkins S, Addy M, Newcombe RG. Dose response of chlorhexidine against plaque and  
557 comparison with triclosan. *J Clin Periodontol* 1994; **21**: 250-255.

558 37 Hoffmann T, Bruhn G, Richter S, Netuschil L, Brecx M. Clinical controlled study on  
559 plaque and gingivitis reduction under long-term use of low-dose chlorhexidine solutions  
560 in a population exhibiting good oral hygiene. *Clin Oral Investig* 2001; **5**: 89-95.

561